Proton Nuclear Magnetic Resonance Characterization of Resins from the Family Pinaceae

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Proton magnetic resonance spectra were recorded for solutions of resinous materials harvested from 82 species in seven genera of the gymnospermous plant family Pinaceae. Data were recorded in both one and two (COSY) dimensions. Approximately 11 peaks in the 1D spectra and 10 cross-peaks in the 2D spectra were present in almost all pinacean spectra, providing a familial diagnostic. Some 40 1D peaks or peak clusters and 60 2D cross-peaks or clusters were considered significant and are reported, when present, for all species. Whereas previous solid-state ^{13}C data were diagnostic primarily at the family level, the patterns of 1D and 2D peaks may provide diagnostic information at the genus and species levels. These spectra constitute the first broad use of ^{1}H NMR to study plant exudates in general and to provide taxonomic characterization in particular.

Exudates appear commonly on the surface of plants in response to injury or disease.[1,2] They contain a wide variety of chemical components, including terpenes in resins, polysaccharides in gums, mixtures of both in gum resins, polyisoprene in true latexes, long-chain alkanes in waxes, and polyphenols in lacquers.[3] Exudates may be liquids, waxes, gummy materials, or hard solids. Humans have found numerous practical and esthetic applications for them.[3] Our own unpublished survey has found that at least 600 genera in 160 vascular plant families produce exudates, implying production in possibly thousands of species.[4]

We recently reported that plant exudates in general may be characterized chemically by solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy.[5] Because solubility varies from high to negligible, only the solid-state method permits examination of all classes of exudates, including fossilized varieties such as amber. Solid-state NMR spectroscopy examines all the material used to record the spectrum, so the results are characteristic of the bulk and not just the portion that happens to be soluble or volatile. Our previous study[5] comprised 65 species from 20 vascular plant families. Remarkably, within this relatively small sample of biological families, we found nine distinct classes of resin spectra, each exhibiting a unique and characteristic spectral fingerprint. Moreover, spectra were identical for multiple samples of a given species or, in some cases, of genera. Spectra of gums also showed variability and were entirely different from those of resins. Spectra of gum resins possessed elements of the spectral properties of both gums and resins. Thus the ^{13}C NMR spectra of exudates constituted a unique diagnostic property based on bulk sampling. The spectroscopic results generally followed modern taxonomic classifications.

Although solid-state ^{13}C NMR spectroscopy offers the advantages of allowing potentially all species to be characterized in bulk without regard to solubility, there are a number of disadvantages. (1) In many cases peaks are broad. The magic angle spinning procedure cannot approach the resolution of spectra taken in solution. As a result, many peaks merge and potential spectral distinctions are lost. (2) The ^{13}C method does not distinguish alkene resonances (from carbons of double bonds) from arene resonances (from carbons in aromatic rings). Such functionalities would be important in providing more subtle taxonomic distinctions. This drawback applies to spectra taken of solutions as well as of solids. (3) The ^{13}C experiment is inherently less sensitive than the ^{1}H experiment and consequently requires more sample and longer spectrometer times. Moreover, spectrometers capable of measuring spectra of solids are not widely available. Thus ^{13}C methods are more costly, in terms of both sample requirements and spectrometer needs. (4) Many resin samples are not solid and hence cannot be powdered and placed in the sample cell. Although in theory such materials may be examined, in practice samples that remain sticky or gummy are excluded. (5) Whereas there is a host of two-dimensional and multipulse experiments for ^{1}H NMR spectroscopy, the available set is more restricted for ^{13}C NMR spectroscopy.

In light of these drawbacks to the ^{13}C experiment, we decided to explore the utility of solution ^{1}H methods to exudate analysis. Gums and gum resins, however, are excluded by reason of low solubility. In the case of resins, ^{1}H spectroscopy offers a number of potential advantages, which for the most part mirror in inverse the disadvantages of ^{13}C methods listed above. (1) Spectra taken of solutions normally possess very high resolution, allowing peaks to be resolved to a much greater extent. Whereas distinctions based on solid-state ^{13}C spectra were discerned primarily at the familial level, the higher resolution of ^{1}H spectra may provide distinctions at the generic or species level. (2) The alkene and arene regions are distinct in the ^{1}H spectrum. (3) Proton NMR spectroscopy is much more sensitive because of the larger gyromagnetic ratio of the proton. Spectra then may be obtained when much less sample is available, and spectral throughput is very high. (4) Since materials are dissolved, it makes no difference whether they are sticky, gummy, or dry solids. (5) Finally, the availability of two-dimensional methods enables spectral properties to be defined not only based on chemical shifts but also in terms of structural connectivities.

The primary alternative tool for the examination and characterization of fossil and modern resins is mass spectrometry. Mills,[6] Anderson,[7] Grimaldi,[8] and their co-workers have used pyrolysis gas chromatography with mass spectrometry to study fossil resins. Stacey, Cartwright, and McEwan[9] have used GC-MS to study modern resins related to Mesoamerican copals (Aztec and Mixtec artifacts from the 15th and 16th centuries, in which resinous material was used as an adhesive). The primary diagnostic tool was total ion chromatograms. They characterized 12 species of modern resins from the Burseraceae, Pinaceae, and Rutaceae and succeeded in finding good matches with some of the artifactual resins.

In this initial ^{1}H NMR study, we have decided to survey a single family, the Pinaceae, in order to explore the applicability of ^{1}H spectra to exudate characterization. This very large gymnosperm family of evergreen conifers, which includes pines, firs, spruces, cedars, and other common trees, is one of the richest producers of

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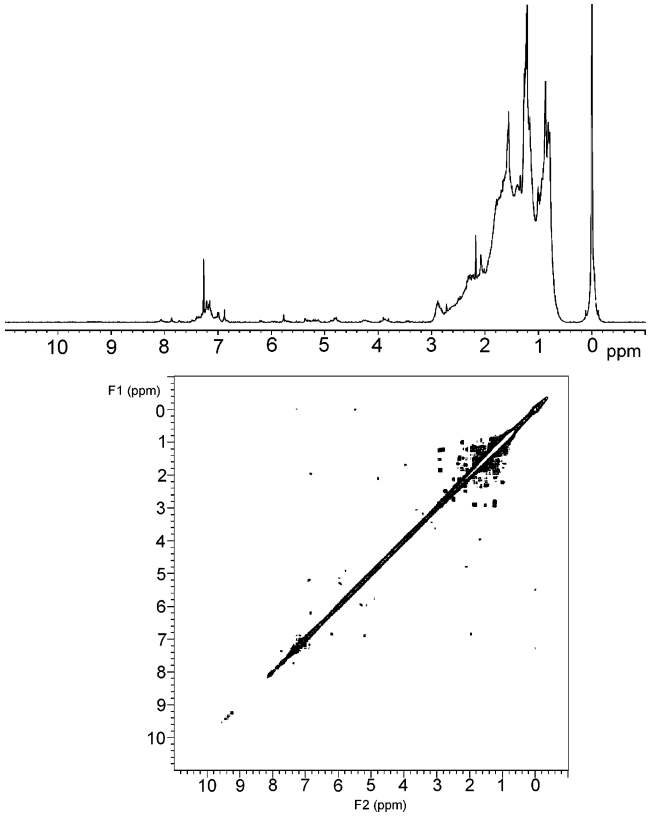

Figure 1. 500 MHz ^{1}H spectrum (top) and COSY spectrum (bottom) of *Abies holophylla*.

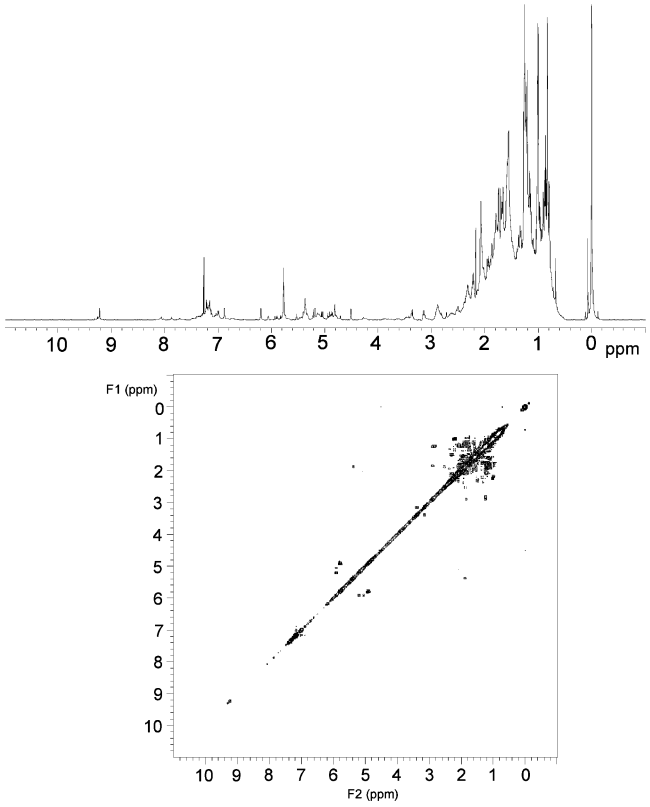

Figure 2. 500 MHz ^{1}H spectrum (top) and COSY spectrum (bottom) of *Abies alba*.

resinous materials. Our study included 82 species from seven of the 11 genera of the Pinaceae (Table S1 in the Supporting Information). We report herein for all species the one-dimensional ^{1}H spectra and the two-dimensional COSY spectra that provide ^{1}H−^{1}H connectivities.[10] Our earlier solid-state ^{13}C study found a single, common fingerprint for the entire family of Pinaceae, based on spectra with normal decoupling and with dipolar dephasing. It was our goal in this study to survey a much larger set of pinacean materials and, in addition, to achieve spectral distinctions at the genus or even the species level, providing a very subtle and potentially powerful taxonomic tool.

Results and Discussion

Genus *Abies*. Eleven *Abies* species, out of approximately 51 extant species worldwide, were analyzed. Seven of these have very similar ^{1}H spectra (*A. chensiensis, A. firma, A. holophylla, A. homolepsis, A. lasiocarpa, A. nebrodensis, A. pardei*), a group we designate *Abies*-1. Figure 1 illustrates the spectra for *A. holophylla*. Two species (*A. alba, A. borisii-regis*) have similar spectra, which are different from the other nine, a group we call *Abies*-2, illustrated by Figure 2 for *A. alba*. Two other species have unique spectra: *A. sachalinensis* (*Abies*-3) and *A. concolor* (*Abies*-4). All major chemical shifts are given in Table S2 of the Supporting Information.

The spectra are dominated by the saturated hydrocarbon alkane region (δ 0.6−2.0). In general, pinacean alkane regions have an undifferentiated envelope that forms the base for various sharp peaks that constitute the largest peaks in the spectrum. The seven samples in *Abies*-1 exhibit, on top of the envelope, three very large sets of peaks, centered at about δ 0.8, 1.2, and 1.6. The δ 1.2 set is the largest. In contrast, *Abies*-2 has three sets of peaks at 0.8, 1.0, and 1.2 of roughly equal size and a smaller set of peaks at δ 1.6. The *Abies*-3 (*A. sachalinensis*) spectrum is similar to that of *Abies*-2 with two large sets of peaks at δ 0.8 and 1.2, a smaller peak set at δ 1.6, and a much smaller peak set at δ 1.0. Finally, the *Abies*-4 (*A. concolor*) spectrum has only two sets of peaks in this region, at δ1.0 (the larger by far of the two) and at δ 1.2.

The region of alkanes with electron-withdrawing substituents such as double bonds and heteroatoms (δ 2.0−4.0) also varies among these groups. This region begins from the low-frequency end as part of the tail of the saturated alkane envelope and grades into separate peaks on the baseline. *Abies*-1 shows three small shoulder peaks usually present at δ 2.1, 2.2, and 2.7 and one small, broad peak at the end of the alkane envelope at δ 2.9. *Abies*-2 shows the same two peaks at δ 2.1 and 2.2, but not the peak at δ 2.7. There is a small peak at δ 2.9 and a characteristic pattern resembling an AX quartet at δ 3.1 and 3.4, also found in one of the *A. pardei* samples. *Abies*-3 (*A. sachalinensis*) shows peaks at δ 2.1, 2.2, 2.3, and 3.7, plus a small peak at δ 2.9. *Abies*-4 (*A. concolor*) exhibits the peaks at δ 2.1 and 3.5 with very small versions of those at δ 2.3 and 2.7.

The alkene region is very weak, except for *Abies*-2, which has modest peaks at δ 5.4, 5.8, and 6.2, with smaller peaks at δ 4.9, 5.2, and 5.5. The arene region in general is more intense than the alkene region for *Abies*-1 and -2, with significant peaks at δ 6.9 and 7.0, a group of peaks around δ 7.2, and smaller peaks at δ 7.9 and 8.1. *Abies*-3 and -4 have only weak resonances in these regions. Extremely small aldehyde resonances are discernible in most of the samples, but *Abies*-2 stands out as the only group with significant peaks, found at δ 9.2.

Two samples of *A. alba*, harvested from different trees, were analyzed. The spectra are visually almost identical, differing only in the presence of two small, sharp peaks at δ 4.5 and 4.8 in one sample. In particular, the AX quartet at δ 3.1 and 3.4 and the aldehydic peak at δ 9.2 are reproduced in both spectra. Two samples of *A. pardei*, also from different trees, differ primarily in the presence of the small AX quartet at δ 3.1 and 3.4 in only one of them. Other differences involve the small, sharp peaks in the alkenic region, a major source of inconsistency throughout this study. We conclude that double-bond functionalities occur variably and are not entirely reliable diagnostic measures.

The COSY spectra are rich in cross-peaks. Many of these occur within the saturated alkane region, between pairs of protons with chemical shifts between δ 0.7 and 2.5. Although many of these peaks no doubt represent important connectivities, we have chosen to ignore them diagnostically. The region forms an overlapping, almost continuous block of peaks. It is possible that cross-peaks may coincide without deriving from the same pairs of protons. Moreover, connectivities within saturated chains are less distinctive than others. Consequently, we have considered cross-peaks only of hydrogens shifted by electronegative groups (EWG), by alkenic protons, and by aromatic protons, within and between these groups and with saturated alkane hydrogens. All important, such cross-peaks are listed in Table S3 in the Supporting Information. Some effort has been expended to characterize the intensities of the cross-peaks, designated in the table as small (S), medium (M), or large (L).

Three cross-peaks are found in all 11 *Abies* spectra and hence characterize this genus as a whole: 1.2/2.8, 1.9/2.9, and 7.0/7.2. Two peaks (1.5/2.9 and 7.4/7.7) are found in all seven *Abies*-1 species plus the single *Abies*-3 species (*A. sachalinensis*). The cross-peak at δ 2.1/4.8 is found in six of the seven *Abies*-1 species but neither in the spectrum of *A. lasiocarpa* nor in those of the other three groups. Numerous cross-peaks are found only in *Abies*-1, but not in all examples. Several cross-peaks are found only in the spectrum of *A. sachalinensis* (*Abies*-3): δ 0.9/4.6, 2.1/5.8, and 5.4/6.2. Several peaks also are unique to the spectrum of *A. concolor* (*Abies*-4): δ 1.0/4.9, 1.3/3.0, and 2.0/5.6. There is no cross-peak unique to both species constituting *Abies*-2. Several cross-peaks are common only to *Abies*-1 and -2: 1.9/5.4, 3.1/3.4, and 4.9/5.8. One cross-peak (δ 2.4/4.9) occurs only in *Abies*-3 and -4, and two (δ 2.5/2.8 and 7.4/7.7) are entirely lacking in *Abies*-2. Thus COSY spectra alone can distinguish almost all the groups.

Most of the cross-peaks represent couplings between peaks cited in the 1D discussion. For example, the four peaks suggested to constitute an AX quartet in both *Abies*-2 spectra indeed generate a cross-peak at δ 3.1/3.4. Interestingly, these peaks are rarely visible in other 1D spectra, but their cross-peaks are evident in several of the *Abies*-1 COSY spectra. The 2D spectra clearly are more sensitive to species differences. There is some ambiguity between cross-peaks at 3.1/3.4 and at 3.2/3.5, which possibly should be combined. As noted, neither cross-peak is present for *A. concolor* or *A. sachalinensis*. Given their chemical shifts, the resonances most likely derive from a pair of diastereotopic protons on the same carbon: $-CH_2-EWG$.

The duplicate samples of *A. alba* gave very similar COSY spectra. The overall patterns are nearly identical, although each sample has one or two cross-peaks not present in the other sample. The duplicate *A. pardei* spectra differ only in small (S) cross-peaks, except for that between the members of the AX quarter at 3.1/3.4.

Genus *Cedrus*. The three analyzed species of this genus, out of four extant species worldwide, have a superficial resemblance in their 1D ^1H spectra to those of *Abies* (see Figure 3 for the spectra of *Cedrus deodara*). Resonances from saturated alkanes dominate the spectra. The alkane envelope is topped by three prominent sets of peaks respectively centered approximately at δ 0.9, 1.3, and 1.6. The sets around δ 0.9 and 1.6, however, have more contributing peaks than for *Abies*. Whereas the spectra of *Abies* tend to have well-defined maxima in these regions, those of the three *Cedrus* species have several peaks of similar intensity. The EWG region also is different. The *Abies* spectra all contained two small peaks in this region at δ 2.0 and 2.1 and a broad peak at δ 2.9. The *Cedrus* spectra all contain the same peak at δ 2.9, but have a large number of additional peaks, from δ 2.0 to 2.4 for *C. atlantica* and *C. deodara* and from δ 2.0 to 2.8 for *C. libani*. The spectra of *C. deodara* and *C. libani* also have an extremely intense peak at δ 3.9, not present in the spectrum of *C. atlantica* or those of *Abies*. The spectra of all three *Cedrus* species have a series of modest

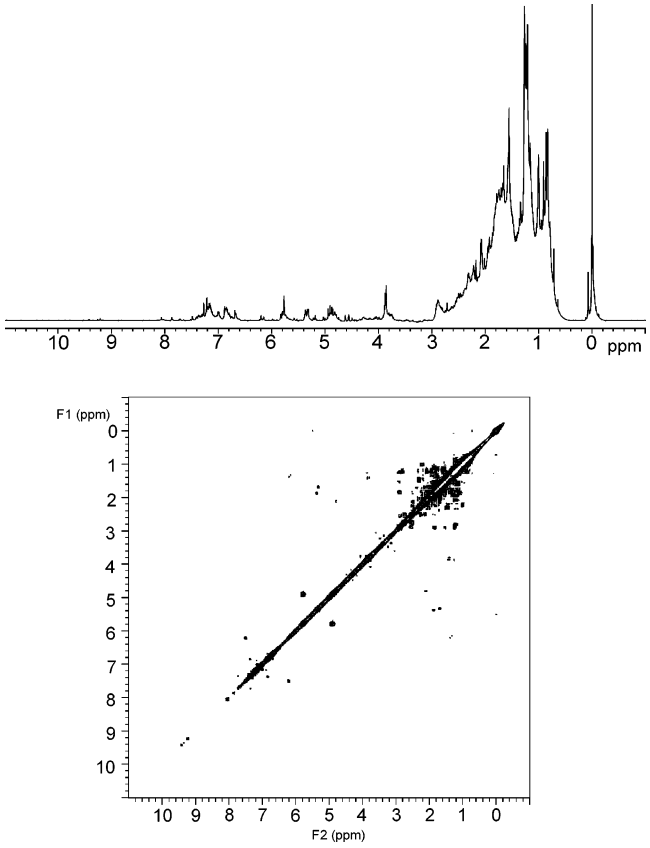

Figure 3. 500 MHz ^1H spectrum (top) and COSY spectrum (bottom) of *Cedrus deodara*.

peaks in the alkene region from δ 4.8 to 6.2. Both *C. deodara* and *C. libani* exhibit a significant, sharp peak at δ 6.7 (alkenic or aromatic), seen only in *A. abies*. The aromatic region is of comparable intensity to that of the alkenic regions, in contrast to *Abies*. Although of stronger intensity, the aromatic region of the *Cedrus* spectra is almost identical to those of *Abies* samples, implying nearly identical aromatic structures in both genera.

Duplicate spectra were taken of *C. libani* from separate trees. The spectra are visually almost identical, save for some differences in intensity.

Numerous COSY cross-peaks are common to all three *Cedrus* species: 1.2/2.8 (11), 1.5/2.9 (8), 1.7/5.3 (4), 1.9/2.9 (11), 2.1/4.8 (6), 2.5/2.8 (9), 4.9/5.8 (9), 7.0/7.2 (11), and 7.4/7.7 (8). The numbers in parentheses are the number (out of 11) of *Abies* species that also exhibit these cross-peaks. Three peaks are found in all 14 species across both genera. These cross-peaks are candidates for general diagnostics for Pinaceae resins. Moreover, they are usually intense (L). The cross-peak 1.2/2.8 is large across all 14 species. While there are differences between the two genera, the resemblances are strongest between *Cedrus* and the *Abies*-1 species.

The COSY spectra of the two samples of *C. libani* are very similar, although there are a few peaks that differ. The cross-peak at 6.2/7.4 is unique for this species (compared with all other *Abies* and *Cedrus* spectra) and is found in both samples of *C. libani*.

Genus *Larix*. This genus is represented by four species, out of approximately 11 species worldwide. All *Larix* samples exhibited an unusual physical characteristic. In contrast to samples from the other genera, these samples produced slightly cloudy solutions in chloroform. The cloudy material settled, aggregated, and floated to the top of the tube overnight but did not represent a large proportion of the total sample. It was filtered off before spectra were taken.

The alkane region as usual is dominated by the large set of peaks at δ ca. 1.2, as illustrated in Figure 4 for *L. russica*. When the

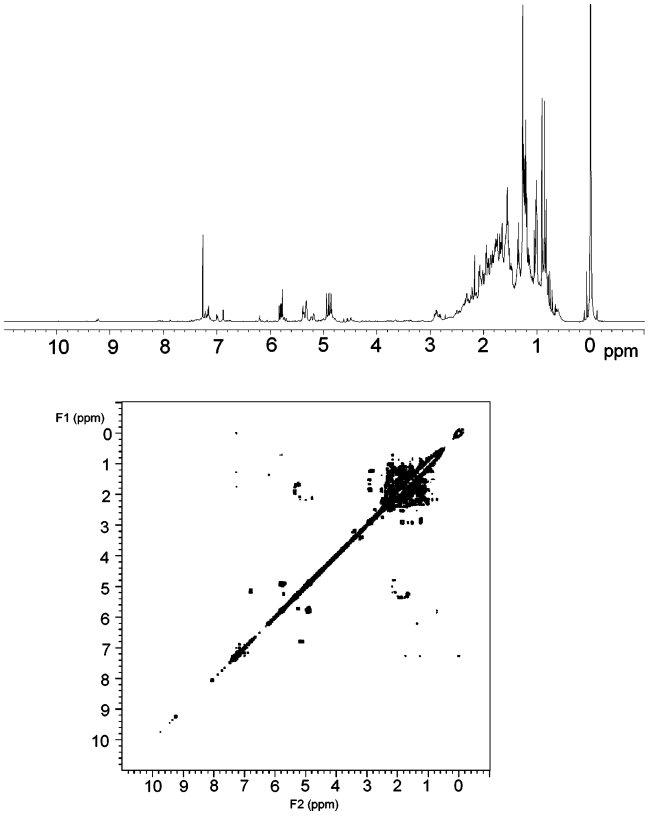

Figure 4. 500 MHz ^{1}H spectrum (top) and COSY spectrum (bottom) of *Larix russica*.

entire alkane region is considered, the spectra of *L. russica* and of *L. laricina* are very similar, and those of *L. decidua* and of *L. kaempferi* are distinct from each of the other three. The resonances at δ ca. 0.9 for *L. russica* and of *L. laricina* consist of three peaks, whereas *L. deciduas* has more and *L. kaempferi* fewer. *L. russica* has a large peak at δ 1.6 and then tails to the baseline by δ 2.5 with no further prominent peaks. The other three species exhibit a broad peak at δ 1.8, which is found neither in *L. russica* nor in any *Abies* or *Cedrus* species. All the species have a peak at δ 2.9, also shared by almost all the *Abies* and *Cedrus* species. There are no important peaks in the EWG region, except for *L. laricina*, which exhibits a unique pair of doublets at δ 3.4 and 3.8 (resembling an AX pattern), not present in other genera either.

All the *Larix* species have moderate alkene absorptions, in contrast to *Abies* and *Cedrus*. The alkenic regions of *L. deciduas* and of *L. kaempferi* are almost identical. All four species exhibit four peaks near δ 4.9. Though weaker, this pattern also is seen in the *Cedrus* spectra and very weakly in the spectra of *Abies*-1. The spectrum of *L. russica* is distinguished by the absence of peaks in the region δ 5.0−5.2, which is populated in the spectra of the other three species (for example, a multiplet at δ 5.1 and a pair of peaks at δ 5.2). All four species have peaks at δ ca. 5.8, but the pattern is distinct for each species, and all four have one or two peaks at δ 5.3−5.4.

The aromatic region is nearly identical for all four species. The pattern is very similar to that of *Cedrus atlantica* and most of the *Abies* species. The aromatic functionalities in Pinaceae genera appear to be highly conserved. The species *L. russica* has a small but distinct aldehyde peak at δ 9.2.

Three different samples of *L russica* were examined from a single source. The spectra are identical, including the small, diagnostic aldehyde peak.

The *Larix* COSY spectra exhibit several cross-peaks in common with *Abies* and *Cedrus*. The cross-peaks at 1.2/2.8, 1.9/2.9, and 7.0/7.2 are present in every species of these three genera. The peaks at 1.5/2.9, 2.5/2.8, 4.9/5.8, and 6.9/7.2 are present in all four *Larix* species and most of the *Abies* and *Cedrus* species (respectively, 11, 12, 12, and 10 out of 14). No other cross-peaks are common to all four *Larix* species. The large peak at 5.2/5.9 is present in the spectra of three of the four species, lacking only in *L. russica*. Several peaks are unique to a single *Larix* species. Thus in principle, all four species may be distinguished from each other and from all species from *Abies* and *Cedrus* by the 2D spectra. The cross-peak at 3.4/3.8 results from the pair of doublets noted in the one-dimensional spectrum of *L. laricina*, now confirmed as an AX quartet and the primary diagnostic of the species.

COSY spectra were recorded for two samples of *L. russica*. Their differences are very minor.

Genus *Pseudotsuga*. This genus is represented here by only a single species, *P. menzieseii*. Its 1D ^{1}H spectrum very closely resembles those of *Cedrus atlantica*, all the *Larix* species, and many of the *Abies*-1 group. Indeed, there is an almost peak for peak coincidence for *P. menzieseii*, *C. atlantica*, *L. russica*, and *A. lasiocarpa*. The alkene region for *P. menzieseii* is intermediate between the strong intensity of *L. russica* and the weak intensity of *A. lasiocarpa*, but the patterns are identical. As in many of these species, the aromatic region is the same. One modest difference between the spectrum of *P. menzieseii* and that of the other species is the low intensity of the trio of peaks near δ 0.9 and the group of peaks near δ 1.0.

The 2D cross-peaks observed for *P. menzieseii* in general are those that are highly conserved throughout the family. There are no unique cross-peaks. Most of the nine observed cross-peaks are found in nearly all the *Abies*, *Cedrus*, and *Larix* spectra. Only 1.7/5.3, 2.1/4.8, and 5.2/5.9 are missing in up to nine of the 18 species from these genera.

Genus *Tsuga*. This genus is represented by two species and is characterized by a very weak alkene region, like the group *Abies*-1. The aromatic region maintains the highly conserved pattern present in the previously discussed four genera. The alkane region of *T. diversifolia* most closely resembles that of *Larix russica*.

We examined three samples of *T. diversifolia*. All three are extremely similar, differing only in very minor aspects. We examined two samples of *T. canadensis*. Although similar in the alkane and arene regions, the alkene region of one sample is much stronger than that of the other. The stronger spectrum is similar to that of *Abies concolor*, in containing a series of sharp singlets. It has a medium peak in the EWG region at δ 3.9, as with *Cedrus libani* and *Cedrus deodara*. This richness of low-intensity peaks may indicate that the sample was contaminated. The sample with lower intensity alkene peaks is very similar overall to that of *Abies pardei* (conserved arene region, very small alkene region, nearly identical alkane region).

The COSY spectra contain the standard pinacean cross-peaks. Otherwise, this genus is not well characterized in the 2D spectrum. The 2D spectra of the three samples of *T. diversifolia* show only minor differences, but there are appreciable differences between the two spectra of *T. canadensis*, mostly in peaks with small (S) intensity.

Genus *Picea*. This genus is large and rich in resin-bearing species. It is represented by 21 species in this study, out of approximately 33 extant species worldwide. Although the 1D spectra show considerable variety, there are some common elements, illustrated for *Picea glehnii* in Figure 5. Every species exhibits the same three peaks in the aromatic region as seen in all previous Pinaceae genera, at δ 6.9, 7.0, and 7.2. Every species has the two small peaks at δ 7.9 and 8.1, also present in all other genera. All spectra contain the broad peak at δ 2.9, which represents the end of the saturated alkane region (or the beginning of the EWG region) and has been observed in all species except *Abies concolor*. Most *Picea* spectra also contain a peak at δ 3.7, which varies from very weak to modest (in *P. glehnii*, *P. likiangensis*, and *P.*

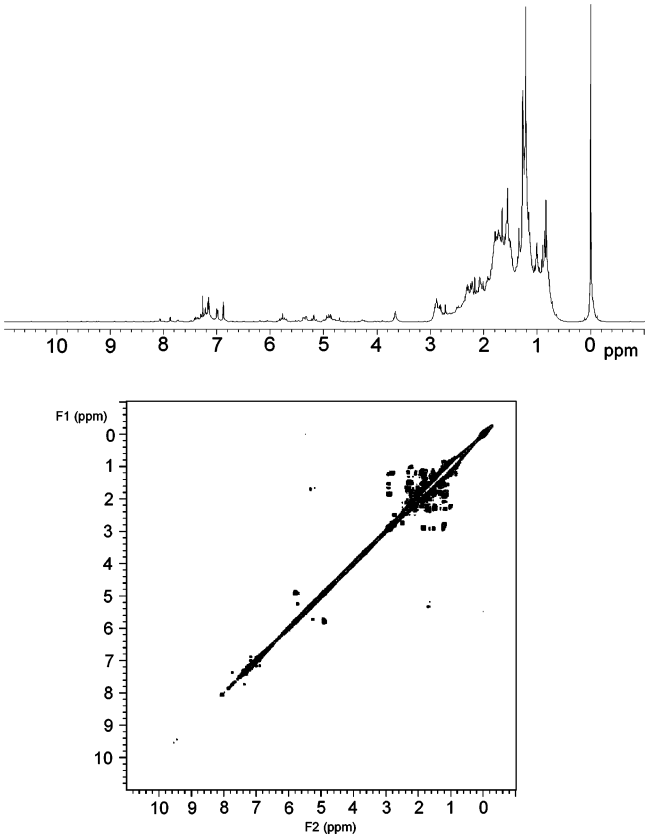

Figure 5. 500 MHz ^{1}H spectrum (top) and COSY spectrum (bottom) of *Picea glehnii*.

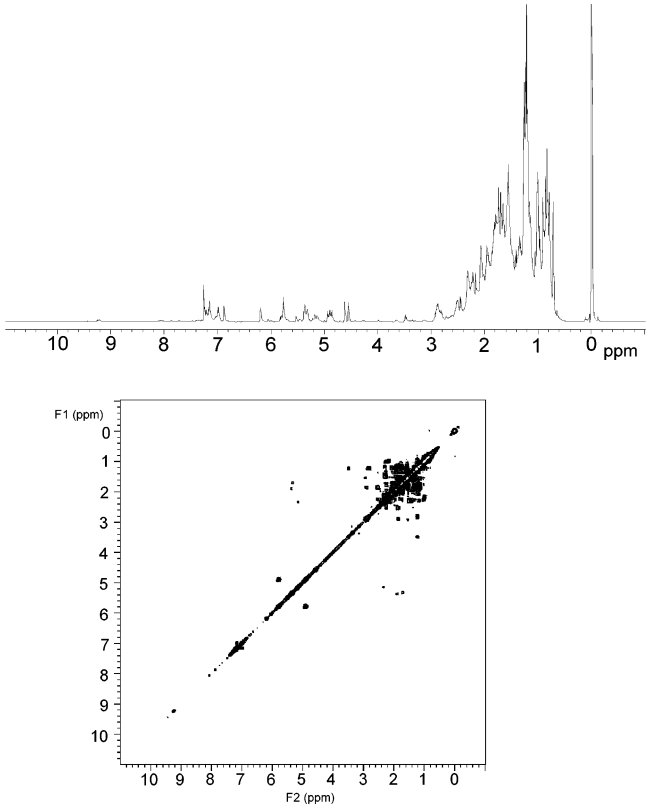

Figure 6. 500 MHz ^{1}H spectrum (top) and COSY spectrum (bottom) of *Picea koyamai*.

maximowiczii). This peak is unusual in the five genera discussed so far, but common in *Pinus*.

As in all Pinaceae samples, the spectra are dominated by the alkane region. The largest peak set as usual is at δ 1.2. There are differences in this region, particularly in the number of peaks at δ 0.8 and 1.0, but there is no discernible pattern. In many cases the region δ 1.8−2.4 forms a plateau of peaks. The envelope descends rapidly to nearly zero by δ 2.6, followed in all cases by the peak previously noted at δ 2.9. Although there are many peaks atop the descending envelope, few protrude much above it, with minor exceptions noted in the table.

The EWG region is generally empty, except for the peak common to the family at δ 3.7. *P. polita* exhibits a strong, sharp peak at δ 3.9, which was seen in only a few of the genera described thus far. A very small version of this peak also was observed for *P. alba*, *P. glauca*, and *P. morrisoncola*.

In general, the alkene region is weak in the *Picea* samples. One common pattern is the peak sets at δ 4.9 (at least four peaks of nearly equal intensity), δ 5.2 (usually a pair of peaks), δ 5.4 (a pair of peaks), and δ 5.8 (a singlet). These sets are common to many other pinacean samples described thus far. The pattern is seen either alone or accompanied by other peaks in all *Picea* species. It is pretty much alone in *P. asperata, P. glehnii, P. likiangensis, P. maximowiczii, P. meyeri, P. montigena, P. obvata, P. omorika, P. orientalis, P. rubens*, and *P. wilsonii.* A peak at δ 4.3 is unique to *P. polita* (this peak also is found in the spectra of both *Tsuga* species as well as all samples of the group *Abies*-1). A sharp peak is found at δ 4.5 in *P. bicolor, P. engelmanii, P. rubens*, and *P. sitchensis*. A pair of sharp singlets δ 4.6 with equal intensity is found in *P. asperata, P. koyamai, P. likiangensis* (one sample, weak), *P. mariana, P. maximowiczii, P. meyeri, P. morissoncola, P. obovata, P. orientalis, P. omorika, P. pungens, P. sitchensis*, and *P. wilsonii*. Alkene intensity is weak in all but *P. koyamai*. A singlet is present at δ 4.7−4.8 in *P. asperata, P. bicolor, P. engelmanii, P. glehnii, P. maximowiczii, P. polita, P. rubens*, and *P. sitchensis*, although relatively large only in *P. bicolor*. A singlet is observed at δ 5.5−5.6 in several species, including *P. abies, P. asperata, P. engelmanii, P. glauca, P. koyamai, P. mariana, P. meyeri, P. montigena, P. morrisonicola, P. obovata, P. omorika* (one sample), *P. polita*, and *P. pungens*, accompanied almost always by another singlet at δ 6.2. This pattern of additional singlets often occurs with enhancement of the singlets at other alkene resonance positions (see Figure 6 for *P. koyamai*). The strong alkene singlets are seen not only in *P. glauca, P. koyamai, P. morrisonicoa, P. omorika*, and *P. pungens* (in one of two samples) but also in *Abies sachalinensis, A. alba*, and *A. borisii-regis* (*Abies*-2 and -3).

Although several species exhibit extremely weak aldehyde peaks, only *P. glauca* and *P. koyamai* have sufficiently large such peaks to be considered significant.

Duplicate samples from different sources were examined for several species. In the case of *P. pungens* two samples were analyzed from the same tree. The spectra of two samples of *P. asperata* have minor differences. The aromatic peaks are weaker in one case, and the alkene group contains weak versions of the extra singlets in the other. The spectra of the two samples of *P. likiangensis* and of *P. orientalis* are nearly identical. The spectra of the two samples of *P. omorika* and of *P. pungens* differ in the same two peaks, the paired singlets at δ 5.5 and 6.2, part of the singlet assemblage found sometimes in the alkene region.

There are eight COSY cross-peaks that are common to all 18 *Picea* species: 1.2/2.8, 1.5/2.9, 1.7/5.2, 1.9/2.9, 2.5/2.8, 4.9/5.8, 6.9/7.2, and 7.0/7.2. The peaks at 1.2/2.8, 1.9/2.9, and 7.0/7.2 have been dominant across the board in this family. The peaks at 1.5/2.9, 1.7/5.2, 2.5/2.8, 4.9/5.8, and 6.9/7.2 are absent only in some *Abies* spectra. Several other cross-peaks are found commonly here as well as in several other Pinaceae spectra: 0.7/5.8 in six (rare, found previously in only two *Abies* species), 1.7/2.9 in 14 (found in six other species not counting *Pinus*), 2.1/4.8 in 11 (common, found in 15 non-*Pinus* species), 2.2/4.4 in 11 (rare, found in only

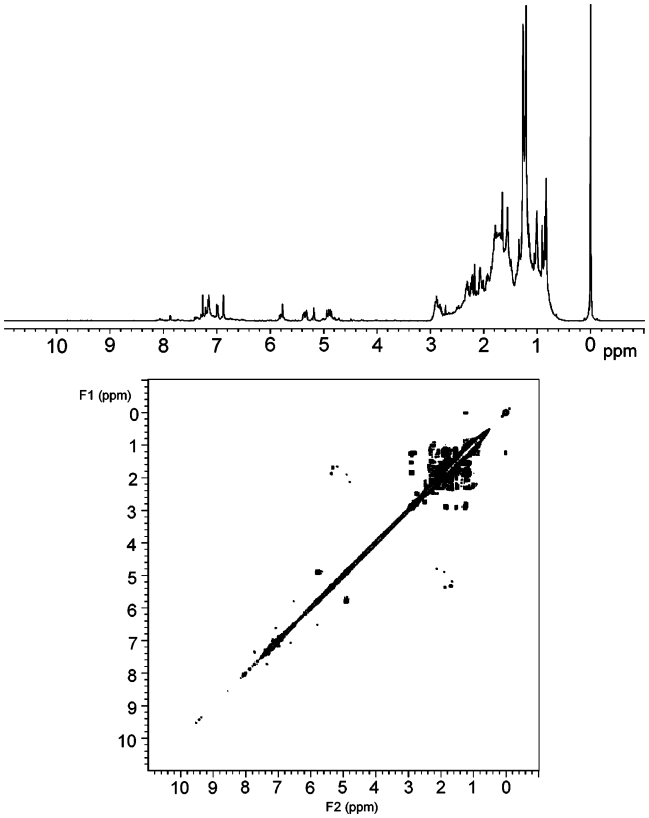

Figure 7. 500 MHz ^{1}H spectrum (top) and COSY spectrum (bottom) of *Pinus halepensis*.

two *Abies* species), 2.3/5.2 in 13 (rare, found in one *Cedrus* and one *Larix* species), 2.9/6.9 in seven (rare, found only in one *Abies* species), 5.0/5.9 in 10 (found in five non-*Pinus* species), 5.2/5.9 in 12 (found in four non-*Pinus* species), and 7.4/7.7 in 15 (common, found in most *Abies* and *Cedrus* species). These peaks thus are indicative of *Picea* but not uniquely so. For the most part each *Picea* species exhibits a unique set of cross-peaks. Some specific peaks are unique to one species, e.g., 1.0/4.9 and 2.4/4.9 to *P. bicolor*, 2.6/3.0 to *P. polita*, and 3.4/3.8 to *P. obovata*, although these occasionally are found in other genera.

The COSY spectra of the two samples of *P. asperata* and of *P. omorika* are very similar, except for a few small (S) peaks. The spectra of the two samples of *P. likiangensis* are identical, except for minor S peaks. The two samples of *P. pungens* show more differences, but they too are mostly in the S peaks.

Genus *Pinus*. This genus is by far the largest in this study, represented here by 39 species, out of over 100 extant species worldwide. There are some elements in the 1D spectra that are common, although not universal, to most other pinacean species described above, including the small, broad peak in the EWG region at δ 2.9, the alkenic peak sets at δ 4.9, 5.2, 5.4, and 5.8, and the three aromatic peak sets at δ 6.9, 7.0, 7.2, and 7.9, as well as the broad alkane envelope generally topped by three large groupings of peaks. It is the variations that are interesting and useful.

The spectrum of *P. halepensis* (Figure 7) is representative in many ways. The alkane region is dominated by the peak cluster at δ ca. 1.2. There also are important clusters at δ ca. 0.8, 1.0, 1.6, and 1.8. The intensity descends in the region δ 2.0−2.6, with numerous peaks of varying intensity. There is a small, sharp peak at δ 2.7 and the universal small peak at δ 2.9 that terminates the alkane envelope. The spectrum of *P. koraiensis* (Figure 8) exhibits some important variations from this pattern. There is a sharp singlet from a methyl group at δ 0.6 (generally found in the range δ 0.6−0.7), found very seldom in other pinacean genera but present in more than half the *Pinus* species examined. The cluster at δ ca. 0.8 has grown in richness and intensity, compared with that of *P. halepensis*. As a third example, the spectrum of *P. pumila* (Figure 9) is significantly different from the spectra of the other *Pinus* samples. The peak cluster at δ ca. 1.2 no longer has the strongest intensity, having been replaced by the cluster at δ 0.8. The peak at

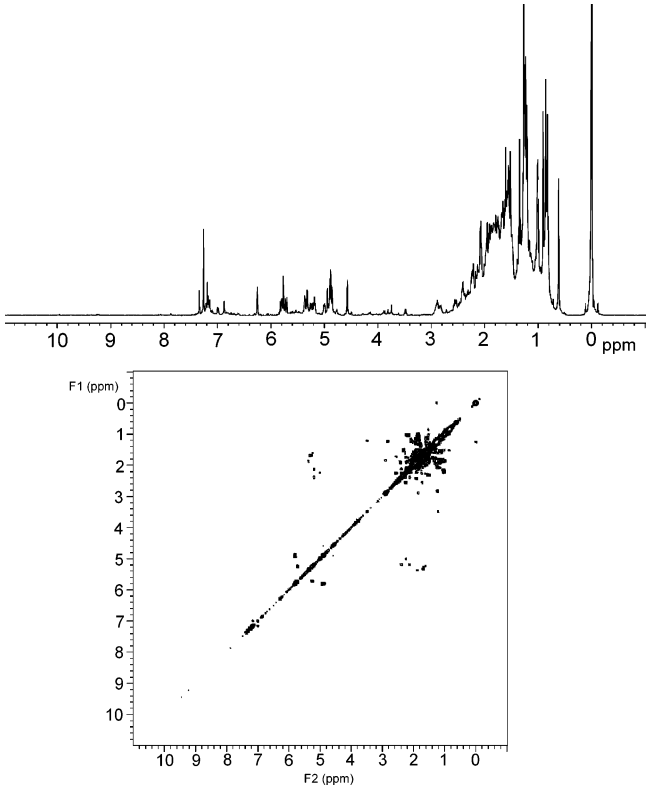

Figure 8. 500 MHz ^{1}H spectrum (top) and COSY spectrum (bottom) of *Pinus koraiensis*.

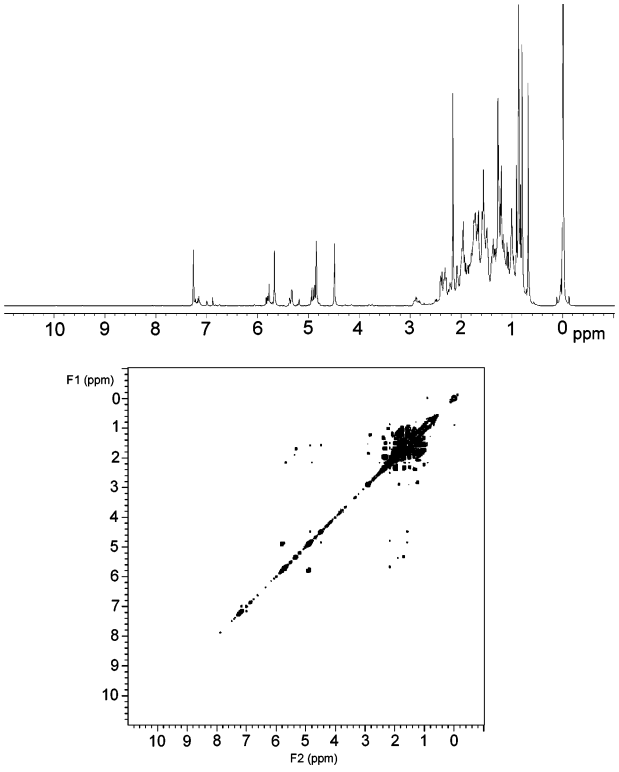

Figure 9. 500 MHz ^{1}H spectrum (top) and COSY spectrum (bottom) of *Pinus pumila*.

δ 0.7 is quite large, and there is a strong singlet at δ 2.2. These three spectra represent the range of *Pinus* spectra in the alkane region.

The EWG region generally is devoid of significant peaks.

Most of the *Pinus* species contain the same four peak sets in the alkenic region that are found in the other pinacean genera, at δ 4.9 (39 out of 39 *Pinus* species), 5.1−5.2 (30), 5.3−5.4 (39), and 5.8 (34). The spectrum of *P. halepensis* in Figure 7 again is representative. These are usually multiplets of variable intensities in these regions. Peaks omitted from Table S2 sometimes were visible but were of very low intensity. In addition, many *Pinus* species have singlets or doublets, also found in other genera, at δ 4.5 (15 *Pinus* species), 4.6 (17), 5.5 (18), and 6.2−6.3 (23) of variable intensity. The peaks at δ 5.5 and 6.2 often are correlated. There are a few other rare alkenic peaks, including those at δ 4.7−4.8 (10) and 5.7 (9). The spectrum of *P. koraiensis* (Figure 8) shows such singlets at δ 4.6 and 6.3, and that of *P. pumila* (Figure 9) at δ 4.5 and 5.7.

Almost all the *Pinus* species retain the highly conserved aromatic region of three peak sets at δ 6.9, 7.0, and 7.2, although the peaks can be quite weak, as in *P. pumila* (Figure 9). An additional singlet at δ 7.4 is seen in some cases. The characteristic peaks at δ 7.9 and 8.1 are reliable but always extremely weak. The aldehyde region usually is empty. Occasionally there are small peaks at δ 9.2 or 9.8 (*P. contorta*, *P. elliotti*, *P. hunneywelli*, *P. mugo*, *P. ponderosa*, and *P. rhaetica*). The peak at δ 9.8 was not observed in the other pinacean genera.

Duplicates were examined for eight *Pinus* species. The two spectra of *P. mugo* and of *P. schwerinii* and the three spectra of *P. parviflora*, of *P. rigida*, and of *P. wallichiana* are almost identical. Slightly greater differences are seen in the two spectra of *P. strobus*. The spectra for the two samples of *P. contorta* differ mainly in the intensity of the alkene peaks compared with the intensities of the other resonances. Most of the peaks are common to both samples, with some important differences in the alkane region. The strong similarities are reassuring that most spectral observations are characteristic of a particular species, but there is some variability.

Only five COSY cross-peaks are found in all 39 *Pinus* species: 1.2/2.8, 1.5/2.9, 1.9/2.9, 4.9/5.8, and 7.0/7.2. The peak at 4.9/5.8 is a superposition of three overlapping cross-peaks. The peaks and three others were present in all 21 *Picea* species. These other three are common but not universal in *Pinus* as well: 1.7/5.3 (32), 2.5/2.8 (33), and 6.9/7.2 (27). Other peaks common to *Pinus* are 1.9/5.4 (20 *Pinus*, 7 *Picea* out of 21 examined), 2.1/4.8 (27 *Pinus*, 15 *Picea*), 2.3/5.2 (19 *Pinus*, 13 *Picea*), and 7.4/7.7 (17 *Pinus*, 16 *Picea*).

Table S3 in the Supporting Information shows many small cross-peaks that are unusual or unique to individual species. It remains to be seen whether S peaks may be used as reliable diagnostics for species. In no case is an M or L peak unique.

The two samples of *P. mugo* and of *P. schwerinii* and the three samples of *P. parviflora*, of *P. rigida*, and of *P. wallichiana* have nearly identical COSY spectra. The duplicate samples of *P. contorta* have the same COSY spectra except in several small (S) peaks. There are differences between the COSY spectra of the two *P. strobus* samples, caused primarily from a large difference in the overall intensities of the spectra. At the M and L level, the spectra are almost identical.

We included in this study a sample that previously had been identified as being in the genus *Pinus*, without species identification.[11] The sample had been called Manila copal Bical, with the Philippines as its source. The spectrum (Figure 10), however, proved to be entirely different from all others in this study. The overall 1D spectral appearance is neither of *Pinus* nor of other Pinaceae genera. It is lacking, for example, the normally dominant peak at δ 0.8−0.9. The COSY spectrum lacks the universal cross-peaks at 1.2/2.8, 1.5/2.9, 1.9/2.9, and 7.0/7.2 (the fifth universal peak at 4.9/5.8 is extremely small), and it contains numerous peaks found in no *Pinus* spectra. The presence of so many cross-peaks not observed in other samples made it impractical to include the 2D data in Table S3. Although the ^{1}H spectra provide no support for a pinacean source, the ^{13}C spectra previously obtained on a solid sample strongly resemble those of *Pinus* samples.[7] Given the Filipino attribution (where *Agathis* normally produces copal), we previously noted that it is likely that either the source or the genus assignment was wrong. It also is possible that the material is a botanical mixture. We hope to find spectral similarities with other exudates as we examine other vascular families.

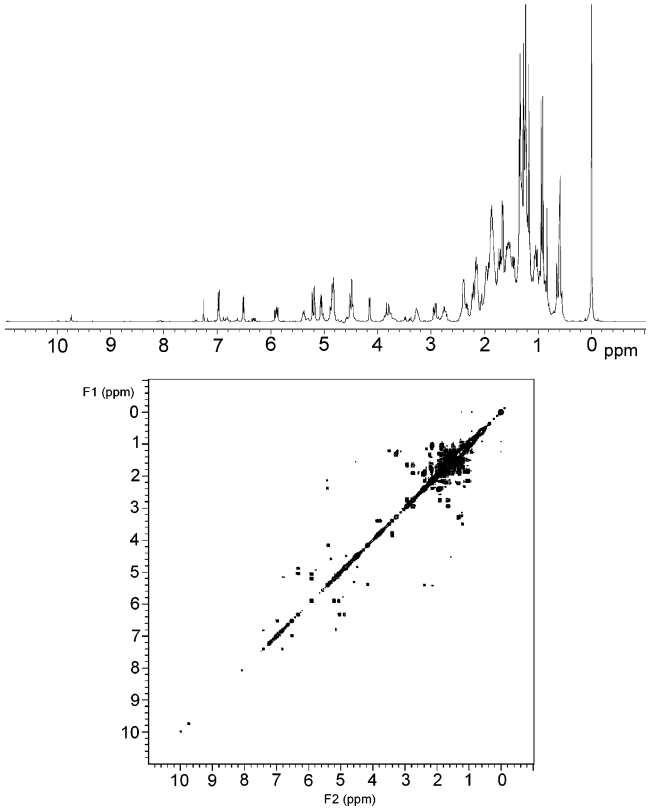

Figure 10. 500 MHz ^{1}H spectrum (top) and COSY spectrum (bottom) of Manila copal Bical.

Summary and Conclusions

Several specific 1D peaks are found throughout all genera and species of the Pinaceae. In the saturated region, the three large groups of peaks at δ 0.8−0.9, 1.2−1.3, and 1.6−1.7 are present in almost every species, that at δ 1.6−1.7 lacking only in *Abies concolor*. Such lacunae serve as diagnostics for a species. The peak at δ 1.0 is present in all but 12 species, including all members of the group called *Abies*-1. A fourth set of peaks found around δ 1.8 is present in all but 18 species, including all the *Abies*, *Cedrus*, *Pseudotsuga*, and *Tsuga* species. The small peak at δ 2.9 is present in all spectra except that of *A. concolor*.

The alkene region has four highly conserved groups of resonances at δ 4.9, 5.1−5.2, 5.3−5.4, and 5.8. The peak at δ 4.9 is absent in five spectra from *Abies* and *Tsuga*. That at δ 5.1−5.2 is absent in six species. That at δ 5.3−5.4 is absent in only two spectra, both from *Abies*. That at δ 5.8 is absent in six spectra, all from *Pinus*. In addition to these sets of peaks, the Pinaceae spectra often have various alkene singlets. For example, a pair of singlets very often occurs at δ 4.6.

The aromatic region is nearly identical for every species of the Pineaceae. Three important groups of peaks at δ 6.9, 7.0, and 7.2 are almost universal in all species (one such peak is missing in *Abies concolor*). Two small peaks at δ 7.9 and 8.1 are present in most species. Aldehyde peaks occur in only a few species.

Almost a dozen COSY cross-peaks are found in almost all the Pinaceae samples. As noted earlier, we omitted all cross-peaks between pairs of peaks in the saturated region, with a cutoff at about δ 2.5. Within this rectangular block of cross-peaks, there are numerous overlaps, representing ambiguous connectivities between alkane neighbors. Cross-peaks outside the region, however, are well defined, including between saturated alkanes and those with electron-withdrawing groups (EWG). Four such cross-peaks are found at 1.2/2.8 (M−L, 0), 1.5/2.9 (S−L, 3), 1.9/2.9 (M−L, 0), and 2.5/2.8 (M−L, 8). The numbers in parentheses represent the number of spectra out of 82 in which the cross-peaks were NOT seen. The intensity designations (S small, M medium, L large) represent the predominant observations for these peaks. There are two frequently observed cross-peaks between saturated protons and alkenes, at 1.7/5.3 (S−M, 16) and 2.1/4.8 (S, 24). A cross-peak between two alkene protons at 4.9/5.8 (M−L, 2) is highly conserved, as are two cross-peaks between aromatic protons at 6.9/7.2 (S−M, 16) and 7.0/7.2 (M, 0). Another aromatic cross-peak is observed somewhat less frequently at 7.4/7.7 (S, 34).

This group of 10 cross-peaks can be considered as diagnostic for the family Pinaceae. The exceptions, however, are important. Most of the absent cross-peaks at 1.5/2.9, 1.7/5.2, 2.5/2.8, 4.9/5.8, and 6.9/7.2 are from the genus *Abies*, and most of these are from species other than *Abies*-1. Thus ^{1}H NMR spectroscopy sets *Abies* apart from the other Pinaceae genera studied. The tables contain many peaks that are diagnostic for individual species or for a small number of species. For example, the peak at 3.2/3.5 is found in seven of the 10 *Abies* species but in only seven of the remaining 60 species from the other genera. It remains to be seen to what extent cross-peaks labeled S are significant. They derive from peaks that are small to the point of invisibility in the 1D spectra. Although it could be argued that such small components could be structurally inherent to the species, it is also possible that variable impurities could cause such peaks. Thus our main conclusions are based entirely on the M and L peaks and those that are highly conserved within the family.

Experimental Section

Plant Materials. Samples were collected from or provided by major botanical gardens or arboreta with permission of the institutions, as listed in Table S1 in the Supporting Information. These samples had been authenticated by the curators and always are confirmed at the time of collection. The exudates were removed from the plant surface by hand or with the help of a knife or other sharp object. This protocol does not produce incisions in plants. Small samples (1−5 g) were collected, of which the ^{1}H NMR experiment requires less than 20 mg. Although the material is dissolved in $CDCl_3$ for spectroscopic examination, the experiment is entirely nondestructive, and the exudate may be recovered by evaporation of the solvent. The materials will remain in the laboratory at Northwestern University for continued experiments but can be made available on request.

NMR Spectra. Data were obtained on a Varian INOVA-500 NMR spectrometer operating at 500 MHz. Approximately 50 mg of the exudate was dissolved in ca. 1 mL of $CDCl_3$, the samples were filtered to remove any particulate matter, and the solutions were placed in 5 mm NMR tubes. Spectra were recorded at ambient temperatures without spinning. For 1D spectra one transient was recorded, and for 2D spectra four transients were recorded with 256 increments. Cross-peaks with intensities below that of the first maximized TMS artifact were discarded. Cross-peaks with higher intensities were classified as small (S), medium (M), or large (L) by inspection.

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Supporting Information Available: Table S1 listing species and sources, Table S2 with proton chemical shifts, and Table S3 with COSY cross-peaks for all Pinaceae species. This material is available free of charge via the Internet at http://pubs.acs.org.

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